

DETECTION OF TOXINS USING IMMOBILIZED CARBOHYDRATES AS RECOGNITION ELEMENTS

Miriam M. Ngundi, Chris R. Taitt and Frances S. Ligler

Center of Bio/Molecular Science & Engineering, Naval Research Laboratory, Washington, DC 20375, USA

ABSTRACT

Carbohydrate-protein interactions are important in many recognition processes that occur on cell surfaces. Bacteria, viruses and toxins use these interactions to bind to the host cells and confer pathogenic properties. These include botulinum, cholera, tetanus, diphtheria, and shiga-like (verotoxins) toxins, as well as influenza virus. In this study, we employed immobilized carbohydrate receptors on an array biosensor to detect protein toxins. Two carbohydrate derivatives: N-acetylneuraminic acid also known as sialic acid (Neu-5Ac) and N-acetylgalactosamine (GalNAc) were immobilized on planar waveguides and interrogated with fluorescently labeled cholera and tetanus toxins. Signals obtained were dose-dependent and indicated that the surface density of the immobilized sugars affected toxin binding.

INTRODUCTION

Recent approaches for detecting protein toxins using the concept of protein-carbohydrate interactions have utilized gangliosides as receptors.¹⁻³ However, because the functional (binding) end of gangliosides is composed of oligosaccharide moieties, this approach has not been successful in identifying the specific carbohydrate epitope(s) that are responsible for protein-carbohydrate interactions. Most of the current studies of protein-carbohydrate interactions have employed lectins as the targets for immobilized sugars⁴⁻⁶ and therefore, no extensive information is available regarding the interactions of pathogenic microbes with specific carbohydrate moieties. Moreover, varied combinations of monosaccharides can yield numerous oligosaccharides that could be employed as ligands for protein toxins, bacteria and viruses. Therefore, there is a need for developing array and microarray techniques for screening and studying protein-carbohydrate interactions, in particular, for detection of pathogenic microorganisms that could be of interest as biological warfare agents. Our study focuses on the development of an array of immobilized monosaccharides which we have used to probe the interactions of the sugars with pathogenic protein toxins as well as bacterial cells such as *staphylococcus* enterotoxin B (SEB), *E. coli*, *listeria*, and *salmonella*.

METHODS

Glass slides (waveguides) were cleaned for 30 minutes in 10% KOH (w/v) in methanol followed by copious rinsing with deionized water and drying under nitrogen. The clean slides were then treated under nitrogen with 2% 3-aminopropyltriethoxysilane in 90% methanol/water (containing trace of acetic acid) for 1 hour. The slides were washed thrice with methanol followed by 3 washes with deionized water before drying under a stream of nitrogen and baking at 120°C for approximately 6 minutes. The aminosilane-modified slides were used immediately or stored at room temperature under nitrogen. Immediately prior to attaching monosaccharides onto the waveguides, the aminosilane-derivatized slides were incubated for 30 min at room temperature in 1mM N-succinimidyl-4-maleimidobutyrate (GMBS) in absolute ethanol, followed by three washes with deionized water. The slides were then dried with a stream of nitrogen and patterned immediately.

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For patterning of each slide, a poly(dimethylsiloxane) (PDMS) flow cell was clamped onto the maleimide-derivatized slide and then the monosaccharide derivatives (Figure 1) containing tris(2-carboxyethyl)phosphine hydrochloride, TCEP-HCl, (0.8 molar equivalent) and triethylamine, TEA, (1.8 molar equivalent) in 70% DMSO/H₂O were added into the channels.

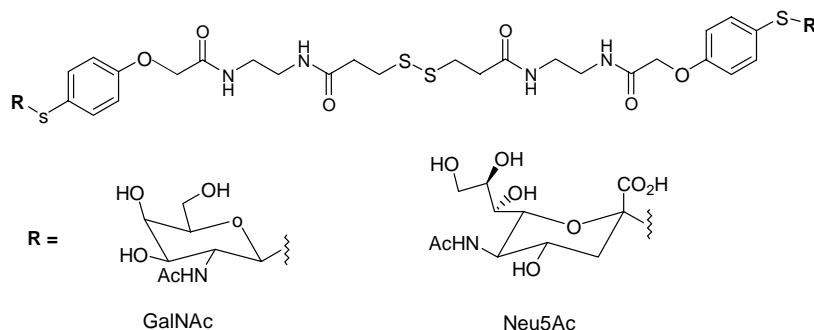


Figure 1: Monosaccharide derivatives used as receptors for toxin detection.

The slides were incubated overnight at room temperature after which the channels were emptied and rinsed successively with 0.5 ml deionized water, 0.2 ml DMSO and finally 1 ml deionized water. The slides were again rinsed with deionized water and blocked with 10 mg/ml bovine serum albumin in PBS containing 0.05% Tween-20 for 30 minutes. After blocking the non-specific sites, the slides were rinsed with deionized water, dried under a stream of nitrogen and interrogated with fluorescently labeled toxins or stored at 4°C. Assays were performed using static method for 1 hr. Binding of the protein toxin to the immobilized carbohydrates was detected using an array biosensor system developed at the Naval Research Laboratory.⁷⁻⁸

RESULTS

TCEP reduces the disulphide bond on the crosslinker between sugar moieties thereby generating two monosaccharides with a thiol-terminated long chain linker. The thiol termini generated in situ react with the maleimide moiety of GMBS on the waveguide giving rise to patterns of immobilized sugars. Direct assays when performed on the immobilized carbohydrate derivatives (sialic acid (Neu-5Ac) and N-acetylgalactosamine (GalNAc)) using Cy5-labeled cholera and tetanus toxins. Our results showed that both toxins bound tighter to immobilized sialic acid than to GalNAc (Figure 2) and that the signals were dose-dependent (Figure 3).

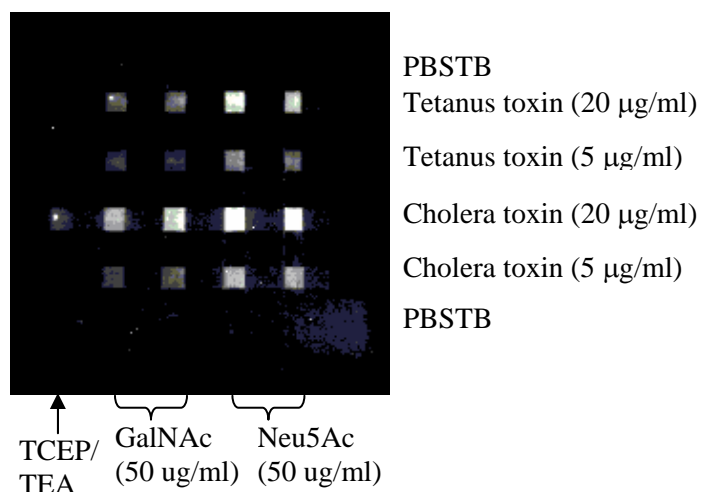


Figure 2: CCD Image of a waveguide after interrogation of immobilized monosaccharide derivative with Cy5-labeled cholera and tetanus toxins.

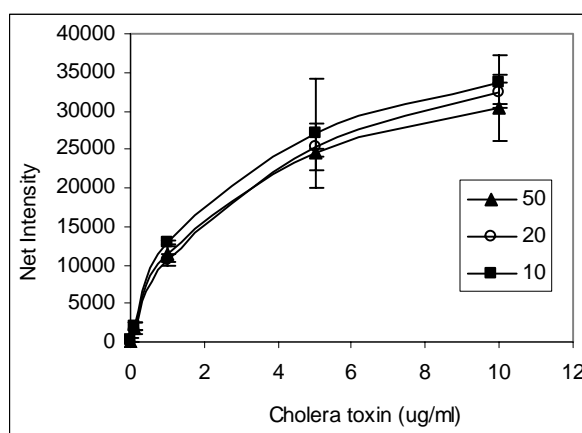


Figure 3: Dose-response curves for the binding of Cy5-labeled cholera toxin to 10, 20 and 50 $\mu\text{g/ml}$ GalNAc.

Lower concentrations of monosaccharides have been employed, and extended research has shown that both toxins could be detected at 100 ng/ml. Real time binding studies, which will be used to determine binding parameters and therefore, the specificities of the sugars towards the various targets are underway.

CONCLUSIONS

We have shown proof of concept that arrays of immobilized monosaccharides could be used to screen and detect protein toxins. In situ generation of the thiol-terminated monosaccharide facilitated the immobilization of the sugars onto the waveguide without the need for a purification step. The assay procedures developed and employed in this study were simple and rapid with a potential of multiplexing the samples. Future experiments include reduction of assay time to 15 minutes, using flow method instead

of static mode, and employing sandwich assays to improve sensitivity. Various oligosaccharides will also be interrogated with varying targets, mainly those of interest as biological warfare agents.

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